**Cell Reports, Volume *42***

## Supplemental information

# Entry receptor LDLRAD3 is required for Venezuelan equine encephalitis virus peripheral infection and neurotropism leading to pathogenesis in mice

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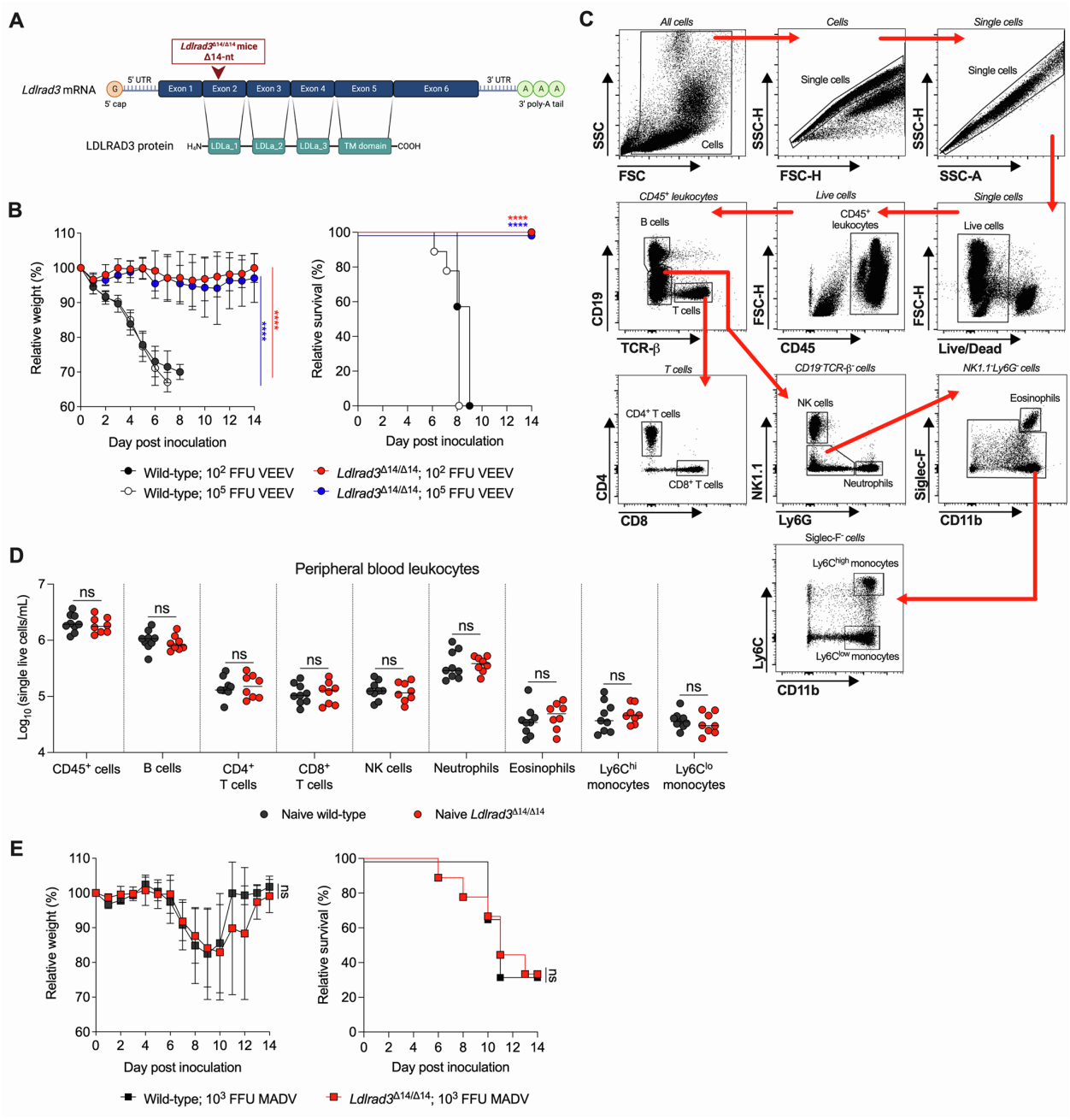


**Figure S1. *Ldlrad3*-deficient mice survive high-dose VEEV challenge but succumb to MADV infection, related to Figure 1.** (**A**) Cartoon schematic of *Ldlrad3* mRNA generated using BioRender. *Ldlrad3* mRNA and corresponding LDLRAD3 proteins with 14-nucleotide frameshift deletion site in exon 2 indicated with a red arrow. (**B**) Seven to 11-week-old male or female wild-type or *Ldlrad3*$^{\Delta 14/\Delta 14}$ mice were inoculated subcutaneously in the footpad with $10^2$ FFU (wild-type, n = 14; *Ldlrad3*$^{\Delta 14/\Delta 14}$, n= 12) or $10^5$ FFU (wild-type, n = 9; *Ldlrad3*$^{\Delta 14/\Delta 14}$, n = 8) of VEEV ZPC738 and monitored daily for weight change (symbols represent mean ± SD) and survival. Red and blue stars indicate a statistically significant difference in weight loss or survival between wild-type and *Ldlrad3*$^{\Delta 14/\Delta 14}$ mice inoculated with $10^2$ FFU or $10^5$ FFU, respectively. Data are from two

independent experiments. (**C**) Representative flow cytometry gating scheme of leukocytes isolated from peripheral blood of a naïve, wild-type C57BL6/J mouse and stained with immune cell markers. (**D**) Analysis of leukocyte cell subsets in the peripheral blood of naïve wild-type C57BL6/J (n = 9) and *Ldlrad3*$^{\Delta 14/\Delta 14}$ (n = 8) mice using flow cytometry. Cells counts are normalized to volume of blood. Data are from two independent experiments. (**E)** Six to seven-week-old male or female wild-type or *Ldlrad3*$^{\Delta 14/\Delta 14}$ mice (n = 9) were inoculated subcutaneously in the footpad with $10^3$ FFU of MADV, a South American lineage of EEEV, and monitored daily for weight change (symbols represent mean ± SD) and survival. Data are from two independent experiments. For weight changes, area under the curve analysis was performed (unpaired t-test). To avoid survivor bias in weight curves, statistical significance was calculated at timepoints when all mice were alive (**B and E, *left***). Survival data were analyzed by log-rank test (**B and E, *right***), and flow cytometry data were analyzed by Mann-Whitney test (ns, not significant; ****$P < 0.0001$).

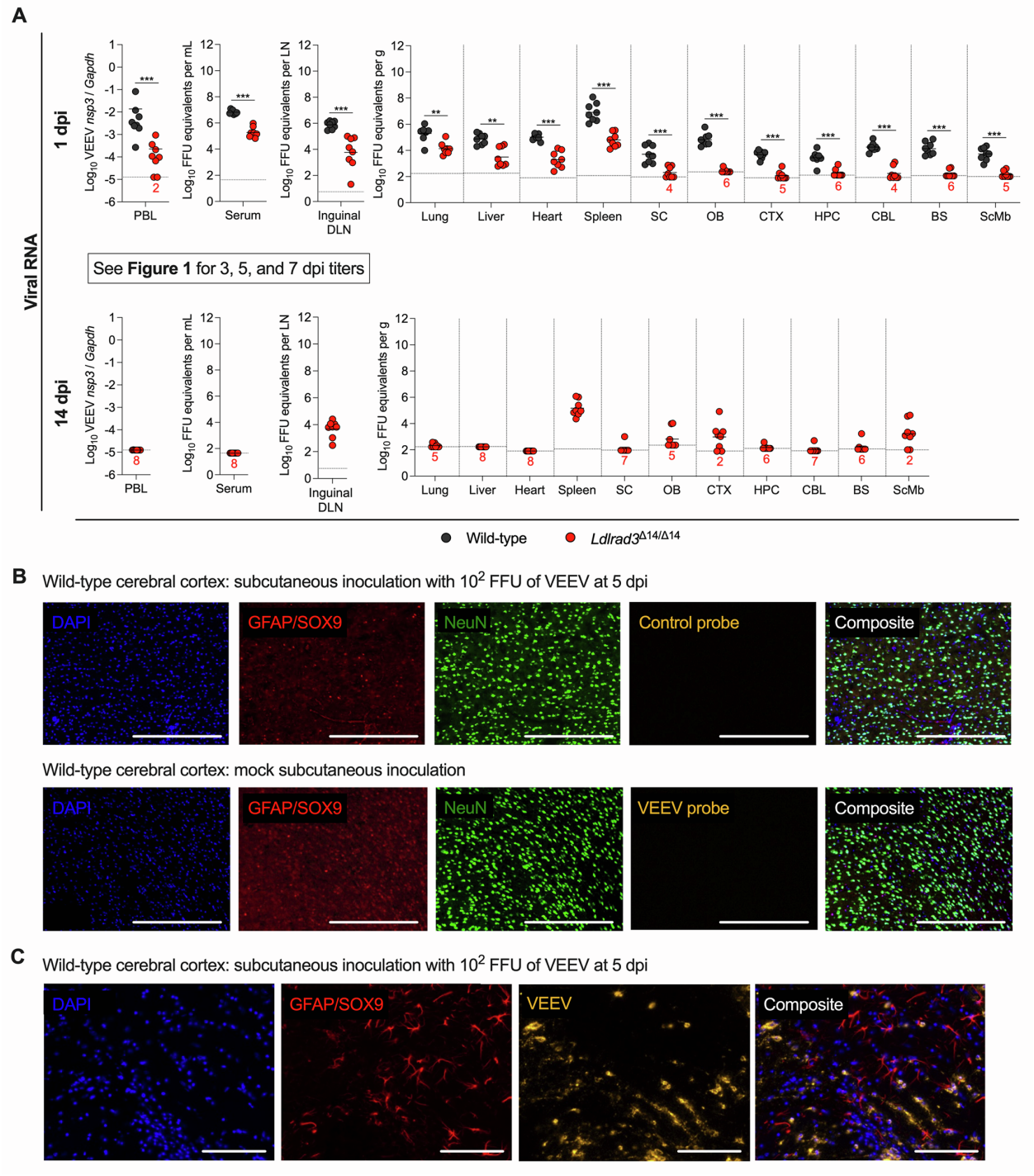


**Figure S2. Viral RNA after subcutaneous inoculation at 1 and 14 dpi and FISH staining controls at 5 dpi, related to Figure 2**. (**A**) Wild-type (n = 7-9) or *Ldlrad3*$^{\Delta14/\Delta14}$ (n = 7-12) mice were inoculated subcutaneously with $10^2$ FFU of VEEV ZPC738. At 1 (for wild-type and *Ldlrad3*$^{\Delta14/\Delta14}$ mice) and 14 dpi (*Ldlrad3*$^{\Delta14/\Delta14}$ mice only), indicated tissues and samples were

assessed for viral RNA by described in **Fig 1** (peripheral blood leukocytes [PBL]); draining lymph node [DLN]; spinal cord [SC]; olfactory bulb [OB]; cerebral cortex [CTX]; hippocampus [HPC]; cerebellum [CBL]; brainstem [BS]; subcortical/midbrain regions [ScMb])**.** Mean values are shown. The LOD for each tissue is indicated by a dashed line, and numbers in black or red enumerate samples with titers at the LOD. Data are from two or three independent experiments per timepoint and analyzed by Mann-Whitney test (***P* < 0.01 and ****P* < 0.001). (**B**) FISH staining controls for VEEV RNA visualization. Images of the cerebral cortex from a wild-type mouse five days after subcutaneous inoculation with $10^2$ FFU of VEEV ZPC738 and stained by FISH with a negative control probe, IHC for cell-specific antigen, and DAPI counterstaining for nuclei visualization (***top panels***). Images of the cerebral cortex from a mock-inoculated mouse stained by FISH with the VEEV-specific probe, IHC for cell-specific antigen, and DAPI counterstaining for nuclei visualization (***bottom panels***) (scale bar: 250 μm). (**C**) Image from the cerebral cortex of a VEEV-infected wild-type mouse at 5 dpi from **Fig 2C** highlighting lack of co-localization between GFAP/SOX9$^+$ and VEEV$^+$ cells (scale bar: 250 μm).

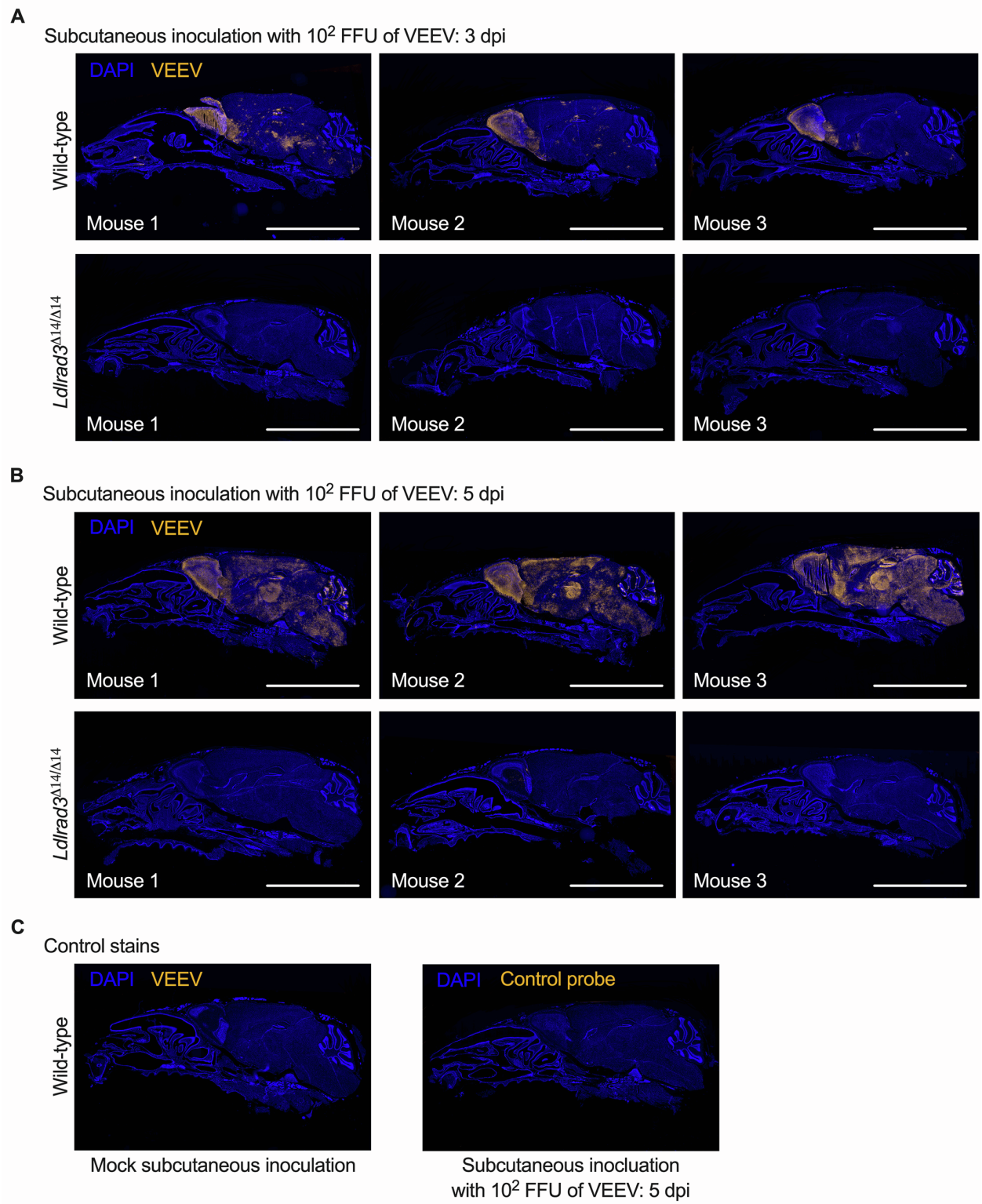


**Figure S3. Fluorescence *in situ* hybridization (FISH) of VEEV RNA in wild-type and *Ldlrad3*$^{\Delta 14/\Delta 14}$ brains at 3 and 5 dpi after subcutaneous inoculation, related to Figure 2.** Images of sagittal skull and brain sections from different wild-type or *Ldlrad3*$^{\Delta 14/\Delta 14}$ mice at 3 (**A**;

n = 4) or 5 (**B**; n = 4) days after subcutaneous inoculation with $10^2$ FFU of VEEV ZPC738 and FISH staining for VEEV RNA and DAPI counterstaining for nuclei visualization. Data are from two experiments. (**C**) FISH staining negative controls for VEEV RNA visualization. Sagittal sections of a wild-type mouse brain 5 days after subcutaneous inoculation with PBS (mock) or $10^2$ FFU of VEEV ZPC738 and staining by FISH with a VEEV-specific probe or negative control probe, respectively, before counterstaining with DAPI for nuclei visualization. Scale bars: 5 mm.

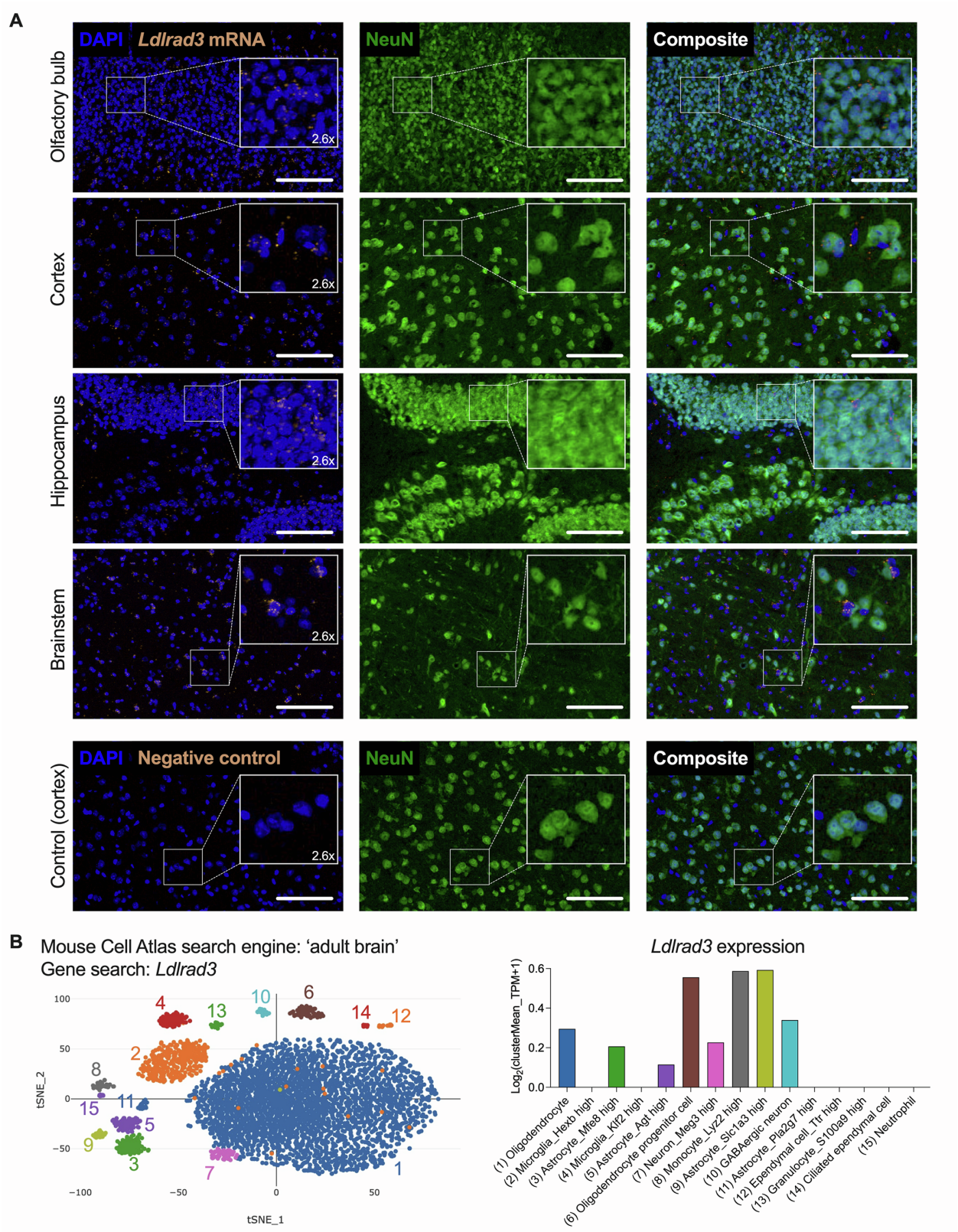


**Figure S4. *Ldlrad3* mRNA expression in wild-type mouse brains, related to Figure 4** (**A**) Representative images from sagittal brain sections of a wild-type C57BL/6J mouse brain (scale

bars: 100 μm) stained with a combination of FISH probe for *Ldlrad3* RNA, or negative control probe, immunohistochemical staining of $NeuN^+$ neurons, and DAPI counterstaining for nuclei visualization. Data are representative of two experiments (n = 3). (**B**) Mouse Cell Atlas Database search results for cell type clusters in the adult mouse brain represented as t-distributed Stochastic Neighbor Embedding (t-SNE) plots (*left*) and corresponding differential expression of *Ldlrad3* RNA in each cell cluster (*right*).

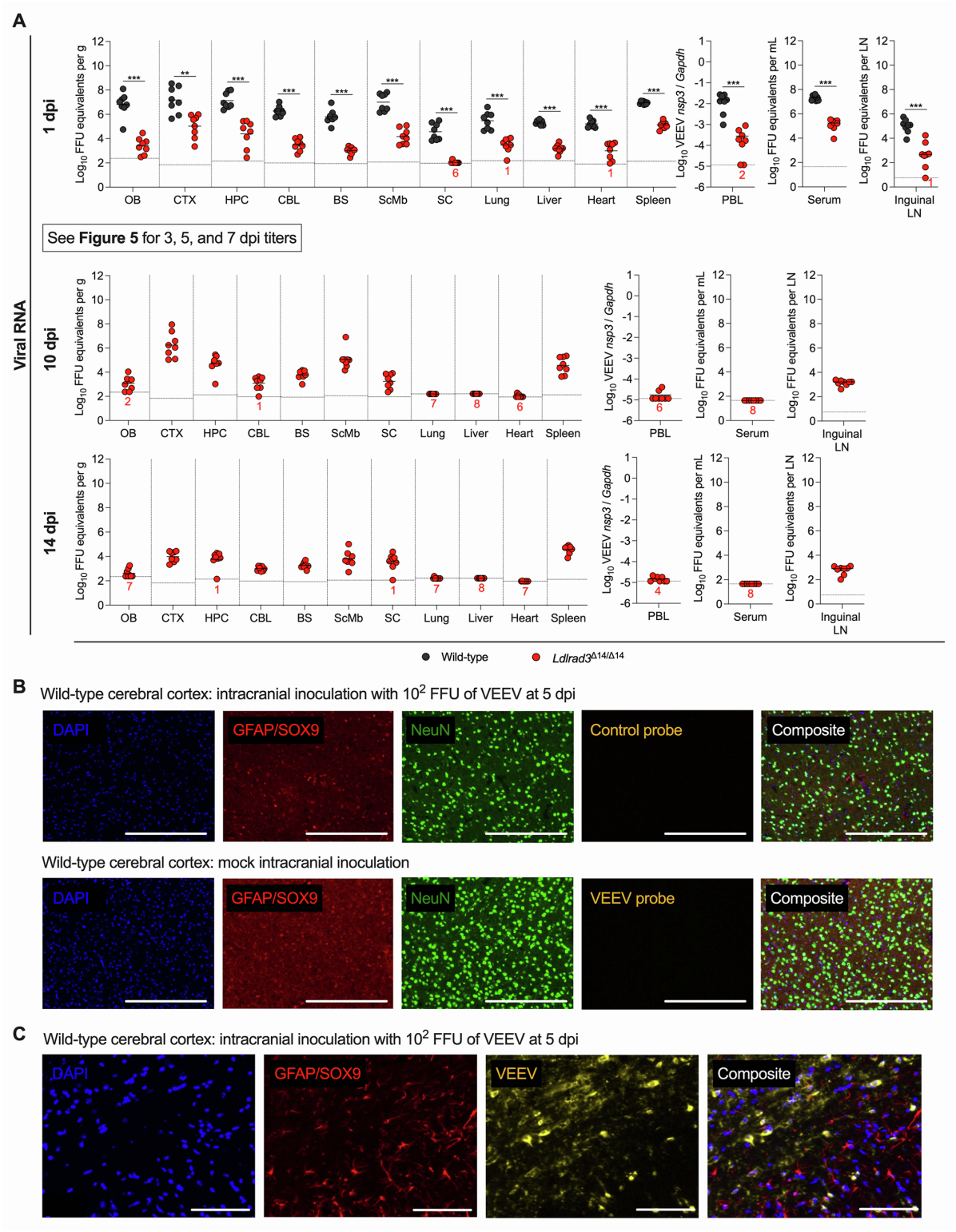


**Figure S5. Viral RNA after intracranial inoculation at 1, 10, and 14 dpi and FISH staining controls at 5 dpi, related to Figure 5.** (**A**) Wild-type or *Ldlrad3*$^{\Delta 14/\Delta 14}$ (n = 8) mice were inoculated intracranially with $10^2$ FFU of VEEV ZPC738. At 1 (for wild-type and *Ldlrad3*$^{\Delta 14/\Delta 14}$ mice) and 10

and 14 dpi (*Ldlrad3*$^{\Delta 14/\Delta 14}$ mice only), indicated tissues and samples were assessed for viral RNA as described in **Fig 1** (olfactory bulb [OB]; cerebral cortex [CTX]; hippocampus [HPC]; cerebellum [CBL]; brainstem [BS]; subcortical/midbrain regions [ScMb]; spinal cord [SC]; peripheral blood leukocytes [PBL]); lymph node [LN]). Mean values are shown. The LOD for each tissue is indicated by a dashed line, and numbers in black or red enumerate samples with titers at the LOD. Data are from two or three independent experiments per timepoint and analyzed by Mann-Whitney test (***P* < 0.01 and ****P* < 0.001). (**B**) FISH staining controls for VEEV RNA visualization. Images of the cerebral cortex from a wild-type mouse five days after intracranial inoculation with $10^2$ FFU of VEEV ZPC738 and stained by FISH with a negative control probe, IHC for cell-specific antigen, and DAPI counterstaining for nuclei visualization (***top panels***). Images of the cerebral cortex from a mock intracranially-inoculated mouse stained by FISH with the VEEV-specific probe, IHC for cell-specific antigen, and DAPI counterstaining for nuclei visualization (***bottom panels***) (scale bar: 250 μm). (**C**) Image from VEEV-infected wild-type mouse cerebral cortex at 5 dpi from **Fig 2C** highlighting lack of co-localization between GFAP/SOX9$^+$ and VEEV$^+$ cells (scale bar: 250 μm).

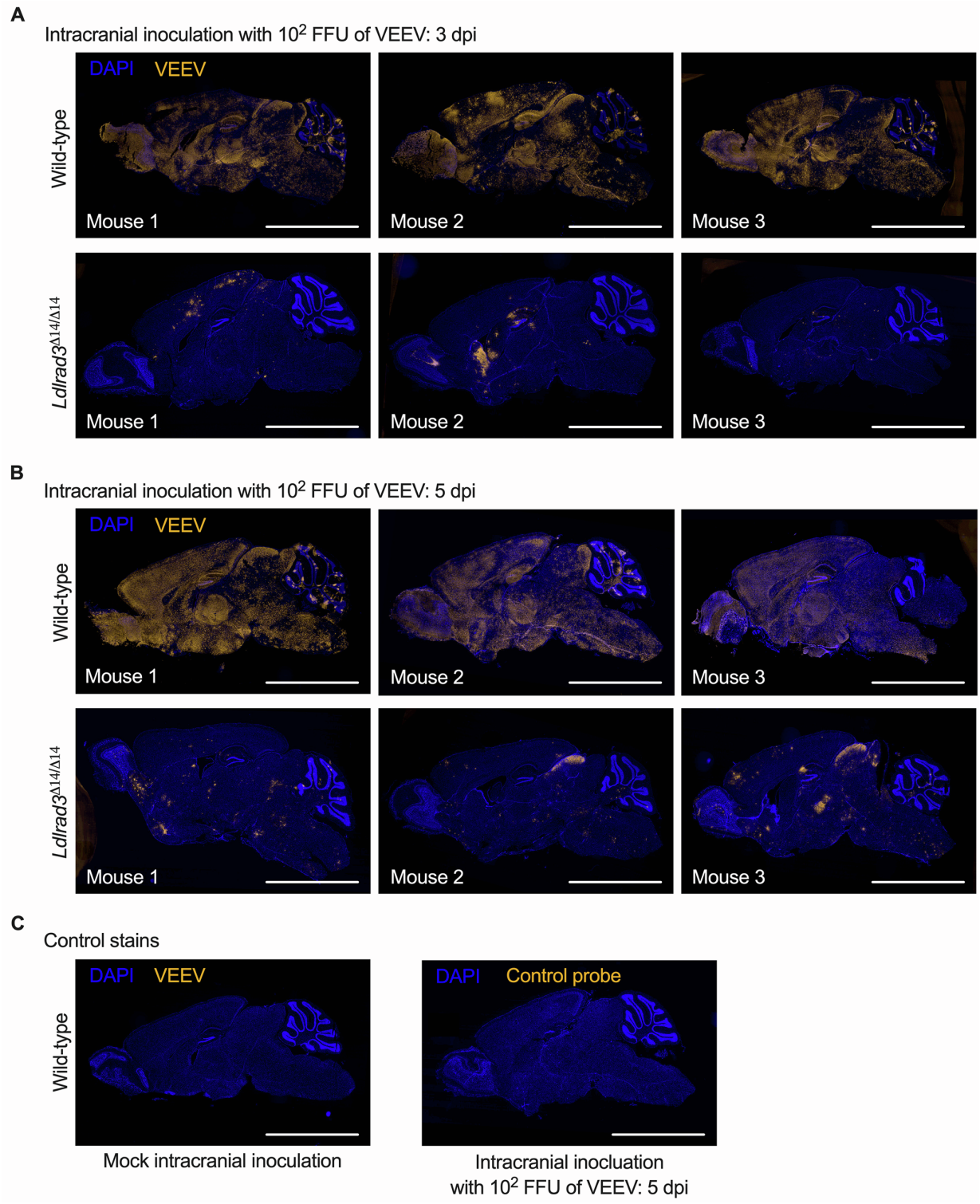


**Figure S6. Fluorescence *in situ* hybridization (FISH) of VEEV RNA in wild-type and *Ldlrad3*$^{\Delta 14/\Delta 14}$ brains at 3 and 5 dpi after intracranial inoculation, related to Figure 5.** Images of sagittal skull and brain sections from different wild-type or *Ldlrad3*$^{\Delta 14/\Delta 14}$ mice 3 (**A**; n = 4) or 5

(**B**; n = 4) days after intracranial inoculation with $10^2$ FFU of VEEV ZPC738 and FISH staining for VEEV RNA and DAPI counterstaining for nuclei visualization. Data are from two experiments. (**C**) FISH staining controls for VEEV RNA visualization. Sagittal sections of a wild-type mouse brain 5 days after intracranial inoculation with PBS (mock) or $10^2$ FFU of VEEV ZPC738 and staining by FISH with a VEEV-specific probe or negative control probe, respectively, before counterstaining with DAPI for nuclei visualization. Scale bars: 5 mm.

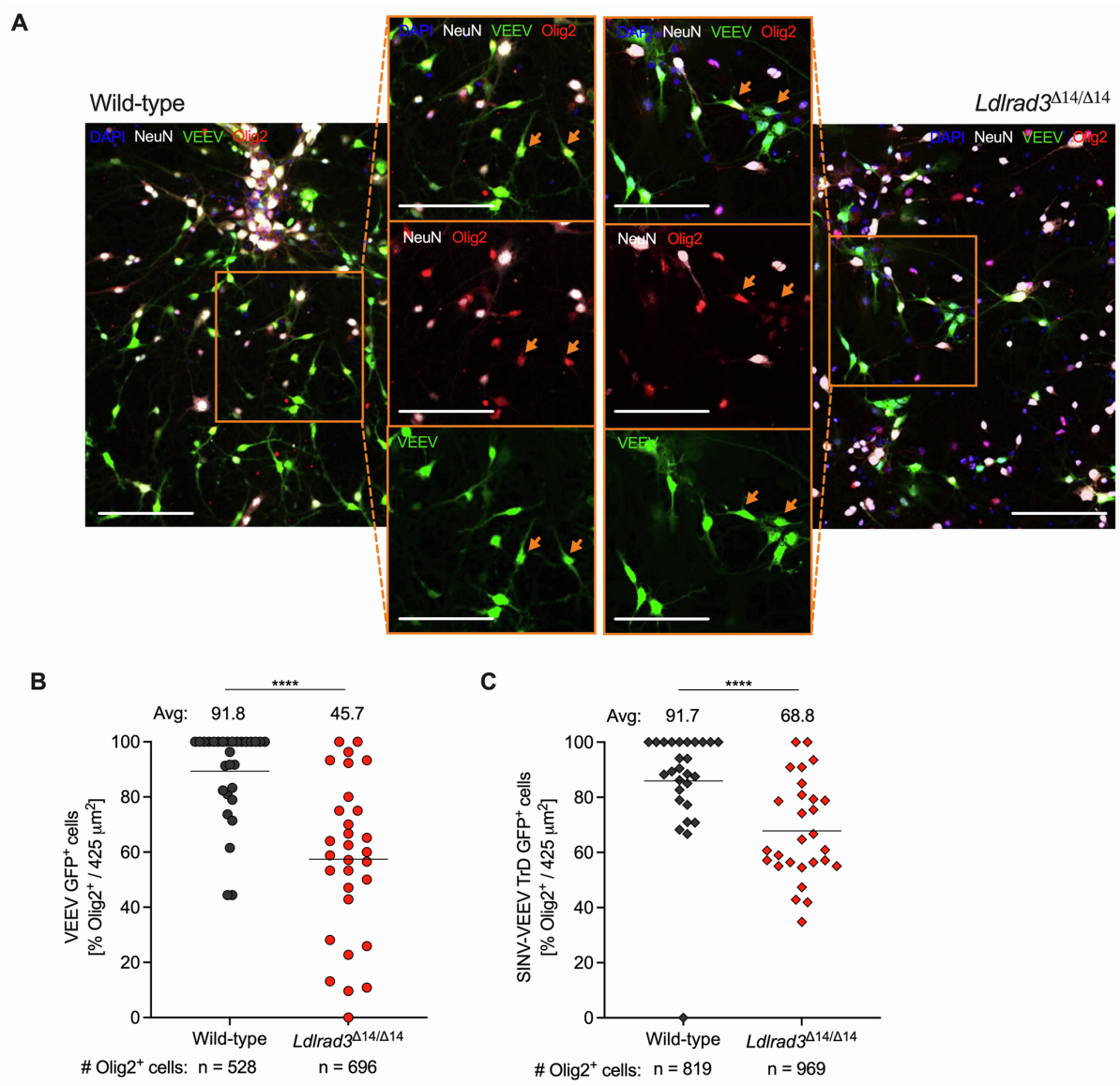


**Figure S7. VEEV infection of Olig2$^+$ cells in LDLRAD3-deficient mixed neuron-glia primary culture, related to Figure 6**. (**A-C**) Immunofluorescence analysis of VEEV-EGFP- or SINV-VEEV TrD-GFP-infected NeuN$^+$ neurons in mixed neuron-glia cultures isolated from E17 embryos of wild-type and *Ldlrad3*$^{\Delta14/\Delta14}$ mice and infected 11 to 14 days after plating at a MOI 20 for 7 h. (**A**) Representative confocal microscopy images of mixed neuron-glia cultures derived from wild-type (left panels) or *Ldlrad3*$^{\Delta14/\Delta14}$ (right panels) mice highlighting nuclei (DAPI$^+$), neurons (NeuN$^+$), oligodendrocyte progenitor cells (Olig2$^+$), and VEEV infection (GFP$^+$). Orange boxes indicate enlarged insets, and orange arrows indicate examples of infected oligodendrocyte lineage cells (Olig2$^+$GFP$^+$ co-localization) (low magnification, scale bars: 100 µm and high magnification, scale bars: 270 µm). Quantification of VEEV-infected Olig2$^+$ cells is represented per image area (425 µm$^2$) as the percentage of Olig2$^+$ cells that are GFP$^+$ for cultures infected with (**B**) VEEV ZPC738-EGFP or (**C**) SINV-VEEV TrD-GFP. The mean percentage of infected oligodendrocyte lineage cells is indicated above each data set, and total number of Olig2$^+$ cells counted is indicated below.

Data are from two independent experiments each with two technical replicates and analyzed by Mann-Whitney test (****$P < 0.0001$).